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REVERSAL OF THE CHANGES IN DNA AND CHROMOSOME STRUCTURE WHICH FOLLOW THE INHIBITION OF UV-INDUCED REPAIR IN HUMAN CELLS

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SUMMARY

The inhibition of UV-induced repair DNA synthesis by hydroxyurea, deoxyadenosine or 1-β-D-arabinofuranosylcytosine is reflected in changes in chromosome appearance (decondensation seen in fixed preparations) and DNA structure (accumulation of unwinding points or single-strand gaps). These changes are rapidly reversed if the inhibition is neutralised by addition of precursors of DNA synthesis. The in vivo organisation of chromosomes apparently survives the prolonged presence of substantial DNA breakage.

The long-held view that certain inhibitors of DNA synthesis such as HU* do not affect repair is no longer consistent with experimental evidence. UV-induced repair DNA synthesis has recently been shown to be inhibited in human and hamster cells by, inter alia, general allosteric inhibitors of ribonucleotide reductase (HU and dA* at high concentration), and by the nucleoside analogue ara C* [1-5]. Inhibition of repair results in changes in both DNA and chromosome structure. The integrity of cellular DNA molecules may be investigated by lysing whole cells in alkali [6], the rate of denaturation (estimated by hydroxyapatite chromatography) depending on the frequency of unwinding points in the DNA. Breaks are introduced in pre-existing DNA during repair of UV-induced lesions, and they accumulate if repair DNA synthesis is blocked [4,5]. Interference with repair DNA synthe-

* Abbreviations: ara A; 9-β-D-arabinofuranosyladenine: ara C; 1-β-D-arabinofuranosylcytosine: BUdR; 5-bromodeoxyuridine: dA; deoxyadenosine: ddT; 2',3'-dideoxythymidine: FUdR; 5-fluorodeoxyuridine: HU; hydroxyurea: PBS; phosphate buffered saline.

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sis also influences the behaviour of DNA when centrifuged on alkaline sucrose gradients [2,3]. There are parallel changes in the organization of mitotic and interphase chromosomes; severe dose-related decondensation of chromosomes (prepared as for standard karyotype analysis) results from incubation following high UV doses, or incubation after lower UV doses with inhibitors of DNA synthesis [2,3,7,8]. We have employed these techniques in studying the kinetics of inhibition of repair DNA synthesis in HeLa cells by various agents. In particular, we consider whether the accumulation of DNA gaps (i.e. nicks or extensive single-stranded regions) during the inhibition of repair DNA synthesis can be reversed at a later stage by supplying precursors of DNA synthesis, or whether the major changes observed represent irreversible degradative processes.

METHODS

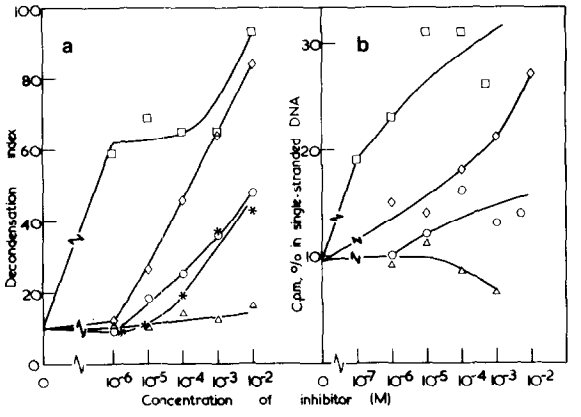
HeLa cells were cultured, labelled with [^{3}H] thymidine if required, synchronised in mitosis by nitrous oxide metaphase arrest, and irradiated with UV at 254 nm as previously described [2].

Chromosome spreads were prepared as described by Tjio & Puck [9], except that the hypotonic medium used to swell the cells was Hanks' Balanced Salt Solution diluted 1:4 with distilled water with a final pH of 7.8. Cells in suspension were incubated in the hypotonic solution at 37°C for 20 min, pelleted and resuspended in freshly prepared Carnoy's fixative. Slides were prepared and flame-dried. Chromosome spreads were scored according to their degree of decondensation, from 0 for normal spreads to 3 for grossly decondensed spreads. Thus, the maximum possible score for the 300 cells counted would be 900 giving a decondensation index of 100.

Alkaline denaturation of DNA was assayed using cells labelled with [^{3}H] thymidine before synchronisation. Following various treatments, cells were suspended in PBS* and 50 µl samples containing 10^5 cells were lysed in alkaline sucrose for 5 min, neutralised, sheared and analysed on hydroxyapatite as described [4].

To study the sedimentation behaviour of DNA (prelabelled with [^{3}H] thymidine) 100 µl samples containing 0.5 x 10^5 cells in PBS were applied to gradients of alkaline sucrose (5-20%) with neutral sucrose overlay [2] and centrifuged immediately for 30 min at 30,000 rpm, 4°C, in an SW50L rotor in a Beckman L2-65B ultracentrifuge. Gradients were fractionated and the DNA profile obtained as described [2].

To detect single-stranded regions in DNA by nitrocellulose binding cells prelabelled with [^{3}H] thymidine and treated in various ways were washed in PBS and centrifuged. The pellet was suspended and lysed in 1 ml per 10^6 cells of 0.01M Tris-HCl, 0.01M NaCl, 0.01M EDTA, 0.1% sodium dodecyl sulphate, pH 8.0, heated at 70°C for 10 min and passed 8 times through a 25G needle. Neutral sucrose gradient centrifugation indicated a weight average sedimentation coefficient of about 28S after shearing, with a variation of 16% between highest and lowest weight average molecular weights. The lysate was digested for 1 h at 45°C with 50 µg/ml of proteinase K. 150 µl aliquots were diluted with 4 ml 6 x SSC (0.9M NaCl, 0.09M Na_3 citrate) and filtered



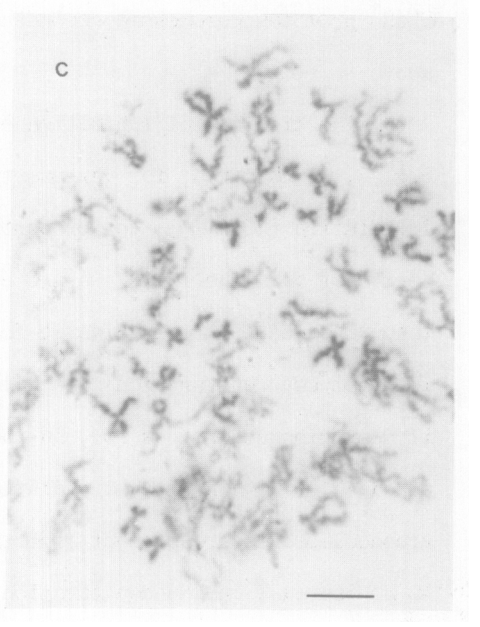


Fig. 1. Effects of inhibitors on DNA and chromosome structure after UV. Mitotic HeLa cells were irradiated with UV (60 Jm^{-2}) and incubated for 1 h at 37°C with ddT (—△—), dA (—○—), HU (—◇—), ara C (—□—) or BUdR (—✳—) at the concentrations indicated. (a) Chromosome decondensation: (b) DNA denaturation in alkali: (c) An example of decondensed chromosomes prepared as in (a), scoring 2. Bar represents 10 μm. ddT was kindly prepared by R. Coulach and its purity established by thin layer chromatography and nuclear magnetic reasonance spectroscopy. It was found to inhibit incorporation of [^{3}H] thymidine by S phase HeLa cells by 90% at 10^{-2}M.

at 1 ml per min through nitrocellulose filters (Sartorius, SM 113, 0.45 μm pore, 13 mm diameter). Filters were washed with 20 ml 6 x SSC and dried. DNA in the filtrates was precipitated with cold 5% trichloroacetic acid, collected on Whatman GF/C 2.5 cm glass fibre filters (W. & R. Balston, Ltd.) and dried. Both filters were counted with 1 ml scintillation fluid (toluene with 4g PPO and 50 mg POPOP/l) on a Packard Tri-Carb Spectrometer.

RESULTS AND DISCUSSION

After 60 Jm^{-2} UV irradiation of mitotic HeLa cells various inhibitors show corresponding activities at the levels of chromosome decondensation and of DNA denaturation in alkali (Fig. 1). Ara C (and also ara A*, not shown) is potent at very low concentrations; HU and dA show an effect only at relatively high concentrations. Surprisingly, ddT*, a chain terminator which inhibits mammalian replicative DNA synthesis [10], has little effect on either DNA denaturation or chromosome decondensation after UV irradiation.

BUdR* promotes chromosome decondensation at concentrations of 10^{-4}M and above.

The kinetics of inhibition of repair at the DNA level are shown in Fig. 2, which gives, for various treatments of HeLa cells following UV irradiation (20 Jm^{-2}), the proportion of DNA denatured after a standard period of alkaline lysis. Ara C establishes its maximal effect more rapidly than HU or dA, and maintains it for longer. However, in all cases the high level of DNA denaturation in alkali is reduced to close to the control level if deoxyribonucleosides (or, in the case of ara C, deoxycytidine alone), are added 30 or 60 min after irradiation and incubation continued. If deoxyribonucleosides are present from the start of incubation (not shown), there is no increase in DNA denaturation.

The sedimentation behaviour of DNA in alkali affords an alternative way of examining changes occurring in DNA as a result of repair and its inhibition (Fig. 3). If irradiated cells are incubated with ara C, the sharp, rapidly moving control DNA peak is replaced with a slow, broad profile probably indicating that the DNA is sedimenting in a more fragmented and denatured form. The addition of deoxycytidine after 30 min incubation with ara C brings about an almost complete return, within an hour, to the fast-sedimenting form of DNA.

The time course of chromosome decondensation in the presence of inhibitors, $\pm$ deoxyribonucleosides, is shown in Fig. 4a and b. UV alone (240 Jm^{-2}) results in chromosome decondensation and this is increased in the presence of each inhibitor. The presence of deoxyribonucleosides from the start of incubation is associated with greatly reduced chromosome decondensation after UV alone or UV with inhibitors (Fig. 4a). Repeated addition of precursors during the incubation period further reduces chromosome decondensation (results not shown). Fig. 4a also shows that FUdR* does not promote decondensation but that BUdR does. The effect of BUdR is reduced if irradiated cells are incubated in the presence of deoxyadenosine, deoxycytidine

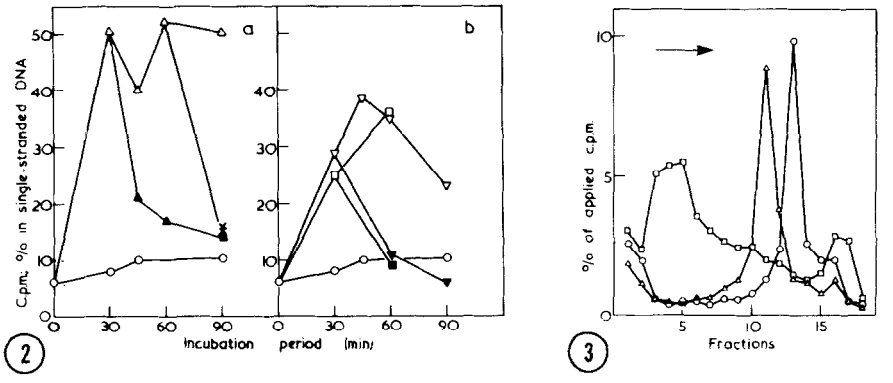


Fig. 2. Kinetics of disruption of DNA structure.
Mitotic HeLa cells were irradiated with UV (20 Jm^{-2}) and incubated for various times at 37°C, and the extent of DNA denaturation in alkali measured. —○—, control (no inhibitor present during incubation); (a) —△—, incubated with ara C at 10^{-4}M or 10^{-5}M (there was no significant difference between effects of these two doses, and the average results of 2 experiments are shown); (b) incubated with dA (2 x 10^{-3}M), —□—, or with HU (10^{-2}M), —▽— (average results of two experiments). Solid symbols indicate the addition of deoxyribonucleosides (deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine) at 10^{-4}M each after incubation for 30 min or 60 min; incubation was then continued. × represents the effect of adding deoxycytidine (10^{-4}M) after incubation for 30 min with ara C (10^{-5}M). The minimal effect of these inhibitors on unirradiated cells in this system has already been shown [4].

Fig. 3. Sedimentation behaviour of DNA from UV-irradiated cells.
Mitotic HeLa cells were irradiated with UV (20 Jm^{-2}). They were incubated at 37°C for 90 min (—○—), or for 30 min with ara C at 10^{-5}M (—□—), or for 90 min with ara C at 10^{-5}M with deoxycytidine (10^{-4}M) added after 30 min (—△—); samples of cells were then centrifuged on alkaline sucrose gradients. The gradient volume is covered by the first 16 fractions. The arrow indicates the direction of sedimentation.

and deoxyguanosine (10^{-4}M) (BUdR taking the place of thymidine). When irradiated cells are incubated for 30 or 60 min, with or without inhibitors, and deoxyribonucleosides (10^{-4}M each) then added, decondensation is greatly reduced; the effects of HU and dA are more readily reversed than those of ara C under these conditions.

We can now attempt to define the relationship between the behaviour of DNA and changes in the chromosomes of cells when repair DNA synthesis is inhibited. We find that mere nicking of mitotic DNA by X-irradiation (up to

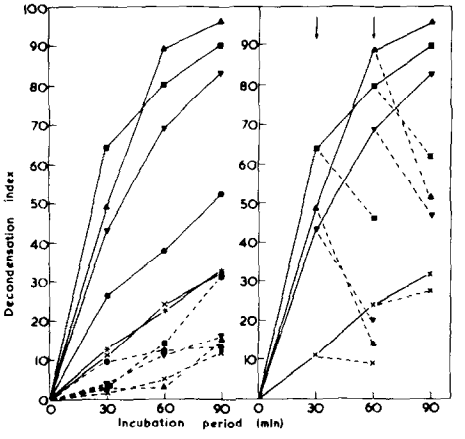


Fig. 4. UV-induced chromosome decondensation in the presence of inhibitors and DNA precursors.
Mitotic HeLa cells were irradiated with UV (240 Jm^{-2}) and incubated for various times at 37°C before making standard chromosome preparations. (a) Decondensation index after incubation with inhibitors (solid lines), or with inhibitors and deoxyribonucleosides (broken lines), present from the time of irradiation. (b) Decondensation index after incubation with inhibitors, present from the time of irradiation (solid lines); after 30 or 60 min, deoxyribonucleosides were added (arrows), and incubation continued for 30 min (broken lines). Deoxyribonucleosides as in Fig. 2 were present at 10^{-4}M each. Control (UV with no inhibitor present during incubation), -×-; incubated with HU (10^{-2}M), -▼-; with dA (2 x 10^{-3}M), -▲-; with ara C (10^{-4}M), -■-; with BUdR (10^{-4}M), -●-; or with FUdR (10^{-5}M), -✳-.

20 Krad at 16 MeV, 1.4 Krad per min) does not result in metaphase chromosome decondensation; neither does the generation of short gaps by the X-ray induced repair process. This suggests that the destabilization of mitotic chromosomes seen when repair DNA synthesis is limited may depend on the accumulation of more extensive regions of single-stranded DNA. Regions of single-strandedness in native DNA can be detected by binding to nitrocellulose filters [11]. Table 1 shows that DNA isolated from UV-irradiated cells incubated with ara C binds to a much greater extent than DNA isolated after various other treatments. This effect is reversed if deoxycytidine is added after 30 min incubation with ara C. (Digestion with single-strand nuclease reduces the extent of nitrocellulose binding to about 5% in all cases). Although binding to nitrocellulose, the ara C - DNA behaved as

Table 1.

Production of single-strand lesions in DNA during UV-induced repair.

Total incubation period (min)	0	30	60	90
Additions	% of DNA bound to nitrocellulose			
None	11	9	13	14 (9)
Ara C (10^{-5}M)	-	36	46	51 (11)
Ara C (10^{-5}M); deoxycytidine (10^{-4}M) added after 30 min	-	-	25	15

Mitotic HeLa cells were irradiated with UV (20 Jm^{-2}) and incubated at 37°C as indicated. DNA was prepared for analysis on nitrocellulose filters; the c.p.m. retained on the filters and passing through were measured to obtain the % bound. Figures in parenthesis represent incubations of un-irradiated cells.

if native on hydroxyapatite, indicating that the amount of single-stranded DNA was small, and this was confirmed by digestion with single-strand specific nuclease which gave a figure of 2.3% for the content of single-stranded DNA. These results are subject to the limitation that enlargement of single-strand gaps may have occurred during the preparation of DNA.

The levels of damage inflicted on DNA in the above experiments are far higher than those known to remove tertiary structure from protein-depleted DNA [12,13]. The fact that these lesions are quickly reversed when inhibitory conditions are removed suggests that the integrity of the chromosome is preserved *in vivo* (though clearly not in the fixed preparations) despite substantial DNA breakage. Specific structural proteins may be involved [14]. However, since temporary inhibition of repair DNA synthesis by HU results in increased cell killing [3,8,15], it is clear that irreversible damage is not entirely avoided. The relationships between inhibition of repair, mutagenesis and chromosome lesions remain to be determined, and it is of interest that deficient excision repair in xeroderma pigmentosum

cells is associated with increased rates of mutation and killing [16]. HU has also been reported to behave as a mutagen [17,18].

Drugs such as HU and ara C have generally been thought not to inhibit repair DNA synthesis [19], and HU has been widely used to facilitate the detection of repair by reducing the background level of replicative synthesis [20-23]. In view of the data discussed in this paper such techniques should be used with caution. There has been some doubt about the mode of action of ara C in mammalian cells [24,25]. The rapid reversal demonstrated here suggests that it acts by competitive inhibition of DNA polymerase or that if incorporated into DNA it can only act as a very leaky chain terminator. The same considerations apply to the inhibitor ara A (data not shown). The inhibitory effect of BUdR described above and its abolition by deoxyribonucleosides are consistent with the recently observed inhibition by BUdR of ribonucleotide reductase [26].

Since completion of this work, Hiss and Preston [27] have presented an alternative explanation of an effect of ara C on UV-irradiated cells, and its reversal, postulating a novel repair system; we believe that their hypothesis is needlessly elaborate.

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